Human erythrocyte metabolism is modulated by the O_2-linked transition of hemoglobin

Irene Messana[a,b], Mario Orlando[a], Loredana Cassiano[a], Lucia Pennacchietti[a], Cecilia Zuppi[a], Massimo Castagnola[a,c], Bruno Giardina[a,b,*]

[a] *Istituto di Chimica e Chimica Clinica, Facoltà di Medicina e Chirurgia, Università Cattolica, Rome, Italy*
[b] *Centro di Studio per la Chimica dei Recettori e delle Molecole Biologicamente Attive, Consiglio Nazionale delle Ricerche, Rome, Italy*
[c] *Istituto di Chimica Biologica, Università degli Studi di Cagliari, Cagliari, Italy*

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Abstract The metabolic behaviour of human erythrocytes has been investigated with particular regard to the effect of their oxygenation state. Experiments performed at high phosphate concentration (80 mM) within the pH range 7.0–7.8 on erythrocytes at high (HOS) and low (LOS) oxygen saturation showed that at any pH value: (1) glucose consumption was independent of the oxygenation state; (2) pentose phosphate pathway (PPP) flux was about 2 times higher in the HOS than in the LOS state. At low phosphate concentration (1.0 mM) the PPP flux doubled in HOS as well as in LOS erythrocytes, whereas the decrease in glucose consumption was more marked in the HOS state. Metabolism of LOS erythrocytes approached that of HOS erythrocytes under the following conditions: (1) erythrocytes having band 3 modified by 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid; (2) CO-saturated erythrocytes. These data support the hypothesis of a modulation of the relative rates of PPP and glycolysis achieved through competition between deoxy-hemoglobin (deoxy-Hb) and glycolytic enzymes for the cytoplasmic domain of band 3.

Key words: Erythrocyte; Red cell; Hemoglobin; Band 3; Glycolysis; Pentose phosphate pathway

1. Introduction

Early studies by Murphy [1] indicated that glucose consumption is higher in deoxygenated versus oxygenated red blood cells and that glucose utilized by the pentose phosphate pathway (PPP) progressively increased as a function of oxygen saturation. Moreover, Hamasaki et al. [2] showed that lactate production and the Embden-Meyerhof pathway (EMP) increased upon erythrocyte deoxygenation. These observations were subsequently confirmed under strict pH control by Rapoport et al. [3]. This unexpected Pasteur effect of human erythrocytes, which are cells without a nucleus and mitochondria, was attributed to complex changes in the activity of various ions, metabolites and cofactors, originating from the higher affinity of deoxy-hemoglobin (deoxy-Hb), as compared to oxy-Hb, for heterotropic effectors, i.e. protons and organic phosphates.

Subsequently, Walder et al. [4] and Chétrite and Cassoly [5] clearly demonstrated that deoxy-Hb displays a higher affinity for the cytoplasmic domain of band 3 (CDB3) than does oxy-Hb. Otherwise, CDB3 is a membrane site also showing high affinity for several enzymes of the glycolytic pathway, such as phosphofructokinase (PFK), aldolase, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and lactate dehydrogenase (LDH) (for a general review see Low [6]). The consequence of this interaction is a general enzyme inhibition [7]. Taking into account all these data, we have recently postulated that the competition between deoxy-Hb and glycolytic enzymes for CDB3 could be responsible for an erythrocyte metabolic modulation [8].

This publication presents results of our investigations which were performed in order to verify the connection between erythrocyte metabolism and the O_2-linked conformational transition (T to R) of Hb, mediated by the competition of Hb and glycolytic enzymes for CDB3.

2. Material and methods

2.1. Materials

^{13}C-1-labelled glucose was purchased either from Sigma (St. Louis, MO) or from CIL (Cambridge Isotope Laboratories, Woburn, MA), ^{14}C-1-labelled glucose was obtained from Amersham (Buckinghamshire, UK). 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) was purchased from Aldrich (Milwaukee, WI). Istagel and phenylethyl amine were obtained from Packard (Meriden, CT). All other analytical grade compounds were purchased either from Farmitalia-Carlo Erba (Milan, Italy) or from Sigma. Erythrocyte samples were offered by informed healthy volunteers aged from 25 to 50 years, under the declaration that they had avoided any drug treatment at least 1 week before sample collection. Samples ranging from 10 to 25 ml were collected in heparinised tubes by arm venipunture and submitted to treatment within 20 min after collection.

2.2. Erythrocyte incubation

Erythrocyte samples were washed 4 times with the following incubation buffers: (buffer A) NaH_2PO_4, 75 mmol/l; K_2HPO_4, 5 mmol/l; NaCl, 18 mmol/l; KCl, 29 mmol/l; (buffer B) HEPES, 25 mmol/l; NaH_2PO_4, 1 mmol/l; NaCl, 86 mmol/l; KCl, 39 mmol/l. Buffers were adjusted to the desired pH by adding an appropriate amount of NaOH and to an osmolarity of 290 ± 5, measured by an Osmostat OM-6020 apparatus (Daiichikagakuco Ltd., Kyoto, Japan). During washing the white blood cells were discarded from the pellet. After washing, 250 μl aliquots of the erythrocyte suspension were transferred to 30 ml plastic vials and an equal volume of 30 mmol/l solution of either ^{13}C-1- or ^{14}C-1-labelled glucose, at a specific activity of 26.6 μCi/mmol in washing buffer, was added.

Before incubation, the plastic vials were closed with rubber caps. When the samples were prepared for $^{14}CO_2$ measurement, they were fitted with a removable central plastic well for $^{14}CO_2$ trapping. A series of samples was submitted to four cycles of in vacuo deoxygenation and nitrogen (ultrapurum) saturation (at a pressure of 760 Torr). These samples represented the erythrocytes at low oxygenation state (LOS). The Hb oxygen saturation was checked in the LOS samples by withdrawing a small portion of the erythrocyte suspension and hemolysing it with deoxygenated distilled water. Hb saturation was determined by spectrophotometric measurements (Cary 3E, Varian, Palo

*Corresponding author. Istituto di Chimica e Chimica Clinica, Facoltà di Medicina e Chirurgia, Università Cattolica, Largo F. Vito 1, 00168 Roma, Italy. Tel.: (39) 6-30154215; Fax: (39) 6-3053598.
E-mail: b.giardina@uniserv.ccr.rm.cnr.it

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Alto, CA), utilising the millimolar absorptivities reported by Zijlstra et al. [9]. The measured percentage of deoxy-Hb was always greater than 80%.

The buffer used to incubate the LOS sample was 0.1 pH unit lower than that used for HOS samples, in order to compensate the Haldane effect occurring during the deoxygenation step [10]. After the deoxygenation procedure, LOS and HOS samples presented an external pH which did not vary by more than 0.02 pH units and which corresponded to an internal pH difference of less than 0.07 pH units [10]. The pH was controlled during incubation by using blank samples containing a solution of unlabelled glucose. At pH 7.4, decrease in pH during incubation was less than 0.03 pH units/h for both LOS and HOS samples with buffer A and 0.02 pH units/h using buffer B. The same samples were centrifuged at $2500 \times g$ for 10 min for estimation of the hemoglobin concentration in the supernatant. Hemolysis which resulted was always less than 0.1%. The incubation was run in duplicate in a thermostated shaker at 37°C.

2.3. $^{14}CO_2$ experiments

The incubation was terminated by adding 250 μl of phenylethyl amine/methanol solution (1 : 1 v/v) through the rubber cap into the central well and 0.5 ml of a 8% solution of perchloric acid to the sample. After 1.5 h, the caps were removed and the phenylethyl amine solution placed into liquid scintillation vials containing 10 ml of Istagel. The vials were counted in a liquid scintillation counter (Tri-CARB 2730, Packard) and the amount of $^{14}CO_2$ produced from each vial was calculated from the number of dpm.

Glucose residue was measured on perchloric acid extracts using a hexokinase diagnostic kit (Sigma). Glucose consumption and $^{14}CO_2$ production were nearly linearly related to the incubation time.

2.4. NMR experiments

NMR experiments were performed on a Gemini 300 apparatus (Varian) using a 5-mm-diameter tube. A capillary containing a 50% D_2O/methanol solution was inserted into the tube. Deuterium from D_2O was used to lock the spectrometer. The methanol signal was used as external standard and its chemical shift was assigned at 49.9 ppm. The measurements were performed at 7.462 MHz and 37.0 ± 0.2°C. The spectra were broad-band decoupled from protons and ranged from 600 to 1200 scans, corresponding to a time period from 18 to 36 min, respectively. The time of incubation was established at the middle point of this interval. A 45° pulse, an acquisition time of 0.8 s and 1 s recovery between pulses were used. A line broadening of 1 Hz was applied before the Fourier transform.

The erythrocyte sample (0.7 ml) incubated at high or low oxygenation state was transferred into the NMR tube. After acquisition, the signals of glucose, lactate, 2,3-DPG and methanol were integrated. The integrals normalised with respect to the external standard were added and compared with the value of the integral of glucose at zero time. From the knowledge of the nanomoles of glucose in the sample at zero time, the nanomoles of each metabolite, as a function of incubation time, were calculated. The lactate production was roughly linearly related to the incubation time.

2.5. DIDS experiments

Band 3 labelling with DIDS was performed according to the method of Jennings and Passow [11]. Red blood cells were washed with buffer pH 7.4 (HOS) and pH 7.3 (LOS) and diluted to 10% hematocrit with phosphate buffer. An amount of a freshly prepared 12 μM DIDS solution was added to the erythrocyte suspensions in order to reach a final DIDS concentration of 0.5 μM. The samples were incubated at 37°C, in the dark, for 1 h. The red cells were washed twice with buffer supplemented with 0.5% BSA and twice with buffer alone, to remove excess reagents. Both erythrocyte suspensions (each 250 μl) were then transferred to 30 ml plastic vials and 250 μl of [^{14}C-1]glucose solution were added. The LOS samples were deoxygenated and incubated following the aforementioned procedure.

2.6. Experiments with carbon monoxide-saturated erythrocytes

Samples prepared in the low oxygenation state (LOS) were flushed twice with an absolute carbon monoxide atmosphere (760 Torr) and then brought under a nitrogen atmosphere. The vials were then incubated at 37°C and one control vial was used to determine the carboxy-Hb content. The carbon monoxide hemoglobin saturation, computed using the millimolar absorptivities reported by Zijlstra et al. [9], was always at a level greater than 90%. The controls for pH values and for hemolysis, done as previously described, provided the same results as those obtained in the case of LOS and HOS samples.

3. Results and discussion

Glucose consumption was measured at high phosphate concentration (80 mM) within the pH range 7.0–7.8 with two sets of samples, representing erythrocytes at high (HOS) and low (LOS) oxygen saturation. The results, reported in Fig. 1, showed that glucose consumption was strongly dependent upon pH, but practically independent ($P > 0.5$) upon the oxygenation state. Nonetheless, the ratio between the $^{14}CO_2$ production and glucose consumption of each sample was significantly higher ($P < 0.0001$) for HOS erythrocytes than for LOS erythrocytes at any pH value (Fig. 2). This ratio corresponds to the fraction of glucose utilized through the PPP and it increased for both oxygenation states as pH decreased. Moreover, the rate of [^{13}C-3]lactate production, measured by ^{13}C-NMR spectroscopy in samples incubated with ^{13}C-1-labelled glucose, was significantly higher ($P < 0.0001$) in LOS erythrocytes (Fig. 3). This rate is linked only to glycolysis. The data revealed that HOS and LOS erythrocytes displayed a significant difference in the glucose addressed towards PPP and EMP and that PPP was about 2 times more active in HOS samples. These results also suggested that different glucose utilization derived from an EMP inhibition not pH dependent.

Experiments performed at a phosphate concentration near to the plasmatic value (1.0 mM) and at a starting pH 7.40 ± 0.02 showed a more pronounced reduction of the glucose consumption in HOS erythrocytes (Table 1). At the same time, the absolute $^{14}CO_2$ production in both HOS and LOS states was about 2-fold that when measured at high phosphate concentration (Table 1). As a consequence, the $^{14}CO_2$/glucose ratio increased in both states, although to a greater extent in HOS erythrocytes.

In order to verify the involvement of band 3 in the oxygen-linked regulation of red cell metabolism, experiments with erythrocytes treated with DIDS, an inhibitor of band 3, were performed. Under these conditions, LOS samples showed a pronounced reduction of glucose consumption,

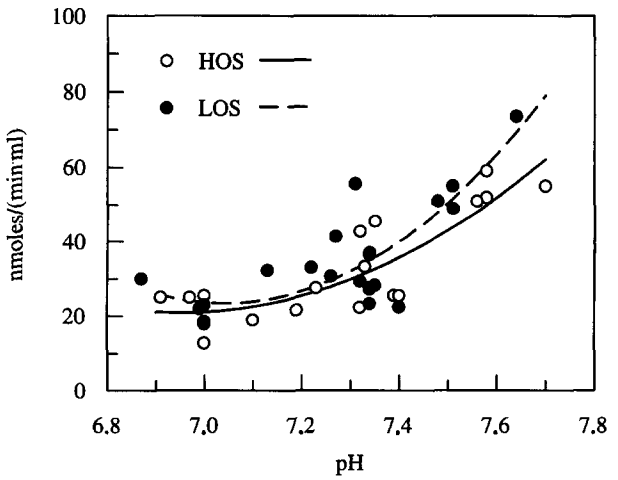

Fig. 1. Glucose consumption of HOS and LOS samples as a function of pH (80 mM phosphate). Difference not significant ($P > 0.5$).

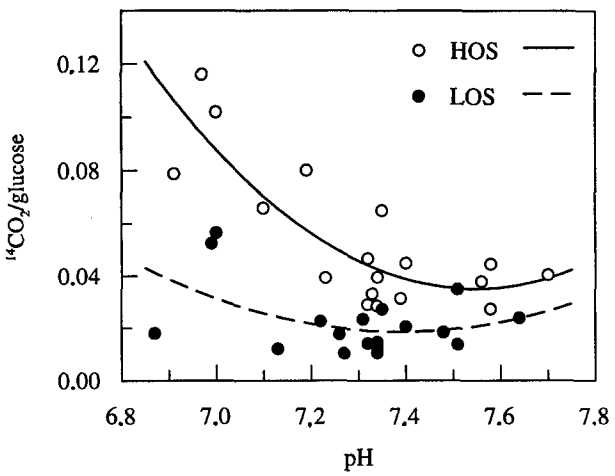

Fig. 2. Ratio between $^{14}CO_2$ production and glucose consumption measured on HOS and LOS erythrocytes, incubated with ^{14}C-1-labelled glucose, as a function of pH (80 mM phosphate). Difference highly significant ($P < 0.0001$).

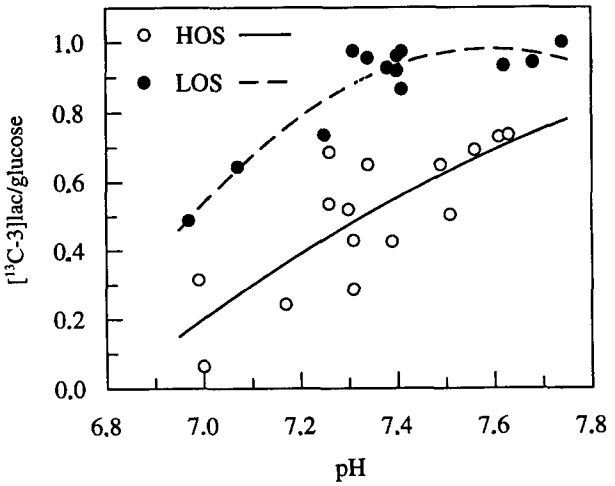

Fig. 3. Ratio between [^{13}C-3]lactate production and glucose consumption measured on HOS and LOS erythrocytes incubated with ^{14}C-1-labelled glucose as a function of pH (80 mM phosphate). Difference highly significant ($P < 0.0001$).

whereas HOS samples were only slightly affected (Table 1). Therefore, a very similar $^{14}CO_2$/glucose ratio was obtained in both samples.

The results obtained agree with the hypothesis that the EMP flux is modulated via a competition between several glycolytic enzymes, mainly phosphofructokinase [12], and deoxy-Hb for CDB3. In fact, in HOS erythrocytes the inhibition of glycolytic enzymes, due to their binding to CDB3, should reduce the glucose flux through EMP. More glucose is then metabolized by the PPP in order to ensure adequate levels of NADPH, necessary to protect the red cell from oxidative stress deriving from the high oxygen erythrocyte load. Conversely, in the LOS state the displacement of glycolytic enzymes from CDB3, induced by the binding of deoxy-Hb, increases the glucose flux throughout the EMP. As a consequence, the ATP and 2,3-diphosphoglycerate production increases (Fig. 4).

To obtain more experimental support for Hb involvement in the oxygen-linked modulation of erythocyte metabolism, PPP rate was measured with samples saturated with carbon monoxide (CO saturation greater than 90%) and incubated at low oxygen pressure. Under these conditions, i.e. maintaining erythrocytes in the LOS state but in the presence of Hb blocked in the fully liganded R conformation, $^{14}CO_2$ production and glucose consumption approached the values measured with HOS erythrocytes (Table 1).

All these results were in agreement with the scheme reported in Fig. 4. In fact, the values obtained on DIDS-treated erythrocytes demonstrated the involvement of band 3 in the metabolic differences observed between oxygenated and deoxygenated erythrocytes, whereas those obtained on CO-saturated erythrocytes revealed the implication of the T conformation of Hb. It can be speculated that the perturbation induced by DIDS on the external domain of band 3 [13] was transmitted at the level of the cytoplasmic domain, which was not able to further release glycolytic enzymes. Whether this was due to impairment of the binding with deoxy-Hb or to increased affinity of glycolytic enzymes still remains to be demonstrated. It is worthwhile to recall that band 3 exists in at least two conformational states, which appear to be under allosteric control [13]. Deoxy-Hb involvement was also sustained by the relatively small differences observed with the erythrocyte metabolism at high phosphate concentration, when compared to low phosphate concentration (Fig. 1 and Table 1). In fact, this observation was in agreement with a competition of the phosphate ion for the same deoxy-Hb site

Table 1
Mean[a] glucose consumption and mean[a] $^{14}CO_2$ production of human erythrocytes

	State	80 mM phosphate	1.0 mM phosphate	DIDS-treated 1.0 mM phosphate
Glucose consumption nmol/(min·ml)	HOS	30 ±3	13 ±2	10 ±2
	LOS	30 ±3	19 ±2	8 ±2
	LOS CO satur.		11 ±2	
$^{14}CO_2$ production nmol/(min·ml)	HOS	1.21 ± 0.14	2.41 ± 0.26	1.95 ± 0.30
	LOS	0.48 ± 0.06	1.20 ± 0.17	1.26 ± 0.16
	LOS CO satur.		1.65 ± 0.22	
$^{14}CO_2$ production/glucose consumption (×100)	HOS	3.7 ±0.5	19.1 ±2	19.5 ±3
	LOS	1.6 ±0.2	6.3 ±0.9	15.7 ±2
	LOS CO satur.		15.1 ±2	

[a]Each mean was obtained by at least three different incubation experiments.
Starting pH: 7.40 ± 0.02.

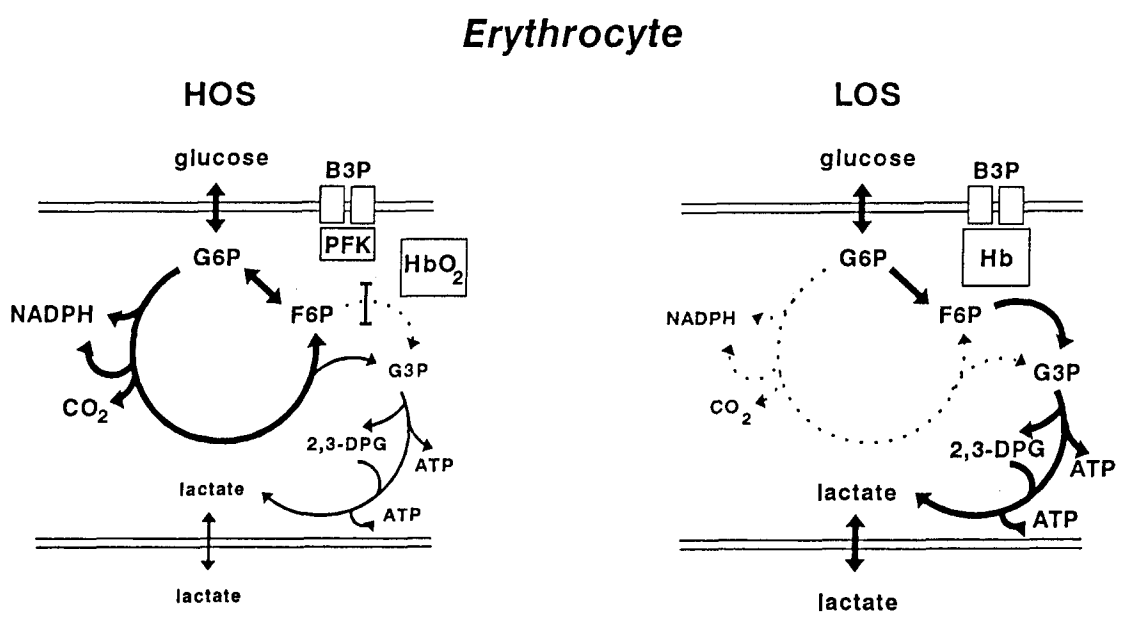

Fig. 4. Simplified scheme representing the modulation of erythrocyte metabolism by the O_2 transition of hemoglobin and its competition with glycolytic enzymes (mainly phosphofructokinase, PFK) for the cytoplasmic domain of band 3 (B3P).

responsible for the interaction with CDB3 [4,5]. This competition should decrease at acidic pH values, due to the phosphate protonation.

In summary, these findings support the hypothesis that erythrocytic metabolic states may be regulated by the free energy linked to the R to T Hb transition [8].

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